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(54) Activating factor of leukocytes

(57) Disclosed are human LECT2a polypeptide having amino acid sequence in the order shown in Sequence ID NOs. from 1 to 5 in the sequence table, which did not include other proteins/peptides and human LECT2b polypeptide having amino acid sequence in the order shown in Sequence ID NO. 6 in the same, which did not include other proteins/peptides, and DNA coding LECT2b sequence.

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Description

BACKGROUND OF THE INVENTION

1. Field of the invention

This invention is concerned with structures of novel human leukocyte activating proteins and DNA encoded the protein. The invention is also concerned with recombinant plasmids and the transformants.

2. Prior art

Neutrophils migrate in response to increasing concentrations of chemotactic factors released from inflammatory sites. Activated neutrophils are recruited from the blood through the vessel wall to the infected site, and phagocytize invading bacteria or viruses. The phagosomes fuse with lysosomes, and the lysosomal enzymes and active oxygen radicals kill the invaders. Thus, the chemotactic factors released from inflammatory sites play an important role in activating neutrophil functions. After reaction by neutrophils in acute inflammation, monocytes and lymphocytes are involved in immune response. In addition to other immune cells including cytotoxic lymphocytes, NK cells and macrophages, neutrophils exhibit a cytotoxic activity against tumor cells in vitro. In their tumorcidal actions, immune cells also migrate to tumor-growing sites, suggesting that tumor cells also produce a chemotactic factor for the immune cells. In this regard, it has been demonstrated that neoplastic cells of mouse and human origin produce chemotactic factors which mediate macrophage infiltration into tumor tissues. Then, we have also identified LUCT/IL-8, which has been constitutively secreted from the carcinoma cell line LU65C (K. Suzuki et al., J. Exp. Med., 169, 1895-1901, 1989). The cells were established from human lung carcinoma tissue infiltrated by many neutrophils. The myeloid leukemia cell line ML-1 and glioma cells also produce LUCT/IL-8 constitutively (K. Suzuki et al., Immunol. Lett. 36, 71-82, 1993). The culture fluids of 97 human leukemia cell lines have been screened to search for a novel chemotactic protein. Then, this invention has been produced.

Neutrophils are seemed to damage cancer cells in addition to macrophages and lymphocytes. Moreover, in some of histological tissues of cancer tissues, are infiltrated with neutrophils. These are believed that neutrophils response to chemotactic factors secreted from cells in these tissues.

Secretion of interleukins and damage of tumor cells are induced by activation of neutrophils, macrophages and lymphocytes. Thus, both natural and acquired immunities are enhanced. When the leukocyte activating factors are purified with large scale and simple procedures, those are used for diagnosis, therapy and determination after treatment. Further, these are also useful for basic research in activating mechanisms of neutrophils, macrophages and lymphocytes.

We have found a neutrophil chemotactic factor, interleukin 8 (LUCT/IL-8) from culture fluid of cells established from human giant carcinoma cells. Then, we purified the factor (protein) and cloned the gene. IL-8 is about 8-kDa plain protein with pI 10.3. Two kinds of protein, 77-amino acid protein and 72-amino acid-protein are classified in IL-8, and possess the chemotactic activities with the same specific activity. Furthermore, IL-8 is a cytokine of inflammation and is detected in serum of patients with chronic inflammation.

However, it has been thought that neutrophil chemotactic factor different from IL-8 is related with interaction between neutrophils and cancer cells.

SUMMARY OF THE INVENTION

The culture fluids of 97 human leukemia cell lines have been screened to search for a novel chemotactic protein. The neutrophil chemotactic protein was detected in a culture fluid of the PHA-activated human T-cell leukemia cell line SKW-3. The protein was purified and its molecular size was determined with sodium dodecyl sulfate (SDS)-polyacryl amide gel electrophoresis (PAGE) as 16 kDa, which is double size compared with that of IL-8. The amino acid sequence analysis revealed that the chemotactic protein is a novel protein, which is designated LECT2a (leukocyte cell-derived chemotaxin 2a). Furthermore, cloning of LECT2a cDNA by polymerase chain reaction (PCR) based on the partial amino acid sequences was carried out. Then, cDNA encoding a protein which had higher homology with LECT2a was isolated. The encoded protein was designated LECT2b.

These results show that the neutrophil activating factor LECT2 involves two kinds of LECT2a and LECT2b proteins, which are identical in leukocyte activation, but different in the amino acid sequence each other. Comparison of amino acid sequence of LECT2a with that deduced from cDNA encoding LECT2b indicates 86% homologous sequence. The amino acid sequences of LECT2a are shown in Sequence ID NO. 1 to 5 in the order from N-terminus. The deduced amino acid sequence of LECT2b is shown in Sequence ID NO. 6 in the order from N-terminus. Sequence ID NO. 7 shows both cDNA nucleotide sequence and deduced amino acid sequence of LECT2b. Amino acid number 58 Val in

the Sequence ID NOs. 6 and 7 can be replaced with Ile. Then, CTC in the nucleotide Sequence ID No. 372 - 374 is ATC. It seems to be due to polymorphism in individuals. When amino acid sequences of LECT2a and LECT2b are compared with each other, there are significant overlaps therebetween. Blank sequence of LECT2a is believed to be higher homology with that of LECT2b, because that molecular size 16 kDa and amino acid component are almost same.

Leukocyte activating factors in this invention are human LECT2 polypeptides having sequences in a Sequence ID NOs. 1 - 5 or sequence in Sequence ID NO. 6 in the sequence table.

This invention is also concerned with genomic DNA which encodes human LECT2b having the amino acid sequence in Sequence ID NO. 6 in the sequence table. Especially, a nucleotide sequence is concerned with the genomic DNA sequence shown in Sequence ID NO. 7 in the sequence table.

This invention is also concerned with the recombinant plasmid encoding human LECT2b having amino acid sequence in Sequence ID NO. 6 in the sequence table. The invention is also concerned with plasmid pMAL-TEV-LECT2b producing fusion protein. The nucleotide sequence encoding LECT2b protein was ligated at down-stream of maltose-binding protein region in pMAL-c vector. Then, the fused protein can be induced by isopropyl-β-D-thiogalactopyranoside (IPTG) treatment. This construct has a TEV protease recognition site which located between maltose-binding protein and LECT2b. The plasmid pMAL-TEV-LECT2b can be obtained from E. coli Mal-LECT2b strain (deposited at the NATIONAL INSTITUTE OF BIOSCIENCE AND HUMAN-TECHNOLOGY, Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, Japan under FERM P-14669 and transferred to international deposition under FERM BP-5302 on November 24, 1995 under the Budapest Treaty). The nucleotide sequence encoding LECT2b protein was ligated at down-stream of glutathione-S-transferase region in pGEX-3X vector. Production of the fused protein can be induced by IPTG treatment. This construct has a Xa protease recognition site which located between glutathione-S-transferase and LECT2b. And, the invention is also concerned with recombinant plasmid DNA encoding human LECT2b regulated with SRalpha promotor, which can highly express LECT2b.

The invention is also concerned transformed cells; especially, obtained by transforming into E. coli, yeast, insect cells, animal cells such as chinese hamster CHO cells, monkey CVI cells, monkey CVI/293 cells, mouse fibroblast cells, mouse C127 cells, mouse 3T3 cells, mouse L-929 cells, human HeLa cells and human SKW-3 cells, which can express the recombinant plasmid encoding human LECT2b having amino acid sequence in Sequence ID No. 6 in the sequence table,

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows determination of molecular size of LECT2a, and molecular size of LECT2a was determined by Tricin-SDS-PAGE with 16.5% monoacrylamide-bisacrylamide: 3% bis/monoacrylamide containing bis.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Leukocyte activation indicates functions of neutrophils, monocytes (macrophages), lymphocytes as follows. The functions of neutrophils and monocytes (macrophages) are as follows; adhesion, migration (chemotaxis), phagocytosis, superoxide production, release of lysosomal proteins/enzymes (degranulation), cellular killing involving tumoricidal activity and production of various cytokines. The functions of lymphocytes are as follows; secretion of immunoglobulins, various cytokines, and expression of various receptors.

The neutrophil activating protein can be purified from a culture fluid of leukemia cells by concentration with CM-Sepharose (trade name, available from Pharmacia Biotech, Uppsala, Sweden) and DEAE-Sepharose (trade name, available from Pharmacia Biotech, Uppsala, Sweden), CM-Sepharose column chromatography, hydroxylapatide chromatography on high performance liquid chromatography (HPLC), and reversed-phase column chromatography on HPLC. For example, SKW-3 cells were maintained in RPMI-1640 medium (trade name, available from GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (available from GIBCO BRL). SKW-3 cells and other leukemia cells were stimulated with PHA-P (trade name, available from DIFCO Laboratories, Detroit, MI) and the leukocytes activating factor described in the invention can be purified this culture fluid using column chromatography etc.

In addition, the neutrophil activating factor can be produced by the transfomant with the recombinant plasmid of the factor. The recombinant plasmid can be constructed with a vector, for example, pMAL-c (trade name, available from Biolab Inc.) or pGEX-3X (trade name, available from Pharmacia Inc.). Animal cells, yeast, and bacteria can be used as transformed cells, for example, chinese hamster CHO cells, monkey CVI cells, monkey CVI/293 cells, mouse fibroblast cells, mouse C127 cells, mouse 3T3 cells, mouse L-929 cells, human HeLa cells and human SKW-3 cells. Cell culture, purification of these activating proteins, construction of recombinant plasmids, transformation, and purification of the protein from the transformant are carried out by common procedures.

EXAMPLES

Explanation of practical example is shown as follows.

1. Procedures for purification of LECT2a Materials were used as follows:
1) 1% bovine serum albumin (BSA, available from Diagnostics, Kankakee, IL) in deionized and distilled water (DDW).
2) R buffer: 50 mM sodium phosphate buffer, pH 7.4
3) R5 buffer: R buffer containing 0.005% BSA.
R1 buffer: R buffer containing 0.001% BSA.
4) CM elution buffer: R1 buffer containing 0.7 M NaCl

1) SKW-3 cells were cultured for 24 hours at 37°C in a 5% CO_2 incubator in a 500-ml glass spinner culture bottle (available from GIBCO BRL) in the presence of PHA-P at a concentration of 5 µg/ml. The culture fluid was harvested by centrifugation at 400 x g for 15 min at 4°C. The supernatant (7 liters) of the culture fluid was divided into 500-ml aliquots and stored at -80°C.
2) Cold DDW (20 liters) was added into the frozen supernatant (7 liters) to thaw the sample. The sample was used for purification of the leukocyte activating factor described in the invention.

Partial purification of LECT2a

1) After the culture fluid was thawed, it was mixed with 200 ml of CM-Sepharose CL-6B (available from Pharmacia Biotech, Uppsala, Sweden) equilibrated with R1 (100 ml packed volume).
2) The mixture was stirred for 4 hours at 4°C.
3) It was filtered through a "KIRIYAMAROHTO SU-95" funnel (trade name, available from Kiriyama, Tokyo, Japan) with 3 sheets of filter paper No. 5B (95 mm in a diameter; available from Kiriyama).
4) CM-Sepharose gel was washed sequentially with 500 ml of cold DDW
5) Then, it was washed twice with 100 ml of R5.
6) The gel was slowly eluted with 50 ml of R5 supplemented with 0.7 M Nacl.
7) This elution was repeated 6 times.
8) The combined eluent was dialyzed against R using Spectra/Por 3 (trade name, available from Spectrum, Houston, TX) which had been rinsed with R5.
9) A DEAE-Sepharose (trade name, available from Pharmacia Biotech) suspension (12 ml packed volume) equilibrated with R5 was added to the dialysate.
10) The mixture was stirred for 2 hours at 4°C.
11) And then it was filtered through a "KIRIYAMAROHTO SU-95" funnel with 3 sheets of R5-rinsed filter paper No. 5B (95 mm in a diameter).
12) The gel was washed with 25 ml of cold R5.
13) The pass-through fraction of the DEAE-Sepharose was applied again to the second CM-Sepharose column equilibrated with R5 (3 ml packed volume in an Econo Column, trade name, available from Bio-Rad Laboratories, Tokyo, Japan).
14) The column was eluted using a linear gradient of 10 ml of R1 buffer and 10 ml of R1 buffer containing 0.7 M NaCl at 4°C at a flow rate of 10 ml/hour.
15) Fractions containing chemotactic activity (Nos. 8-11) were used for partial purified principle.

Complete purification with column chromatography on HPLC

1) The purified samples, which were repeated two times, were combined.
2) The combined sample (2.5 ml, 1.2 O.D.) containing neutrophil-chemotactic activity was applied to a reverse-phase column (trade name, available from Vydac C4 column 304-2151, 4.5 x 250 mm) on HPLC.
3) The column was eluted with 22.5% to 60% acetonitrile containing 0.1% tetrafluoroacetic acid (TFA) at a flow rate of 0.5 ml/min.
4) Finally, the active fractions were rechromatographed with the same reverse-phase column in the same acetonitrile gradient containing 0.05% hexafluorobutyric acid instead of 0.1% TFA.
5) The eluate was pooled and dialyzed twice against 25 mM sodium phosphate buffer, pH 7.4.
6) The eluent was stored at -80°C.

Purification of LECT2a is shown in Table 1.

Table 1. Purification of LECT2a from SKW-3

Purification steps	Volume (ml)	Activity (CTU)	Protein (mg)	Specific Activity (CTU/mg)
Culture fluid	7,000	1.0×10^7	47,200	211
CM-Sepharose CL6B eluate	280	7.7×10^6	3,400	2,265
DEAE-Sepharose CL6B pass-through	300	7.0×10^6	1,800	3,888
CM-Sepharose CL6B fraction	4	2.4×10^4	3.6	6,667
Reverse phase (5TMS-300) fraction*	2	2.1×10^4	0.068	6.2×10^5

*This step represents the results of two chromatograms in different condtions.

Determination of molecular size of LECT2a

Molecular size of LECT2a was determined by Tricin-SDS-PAGE with 16.5% monoacrylamide-bisacrylamide:3% bis/monoacrylamide containing bis. The result is shown in Figure 1. This result indicates that the molecular size of

LECT2a is about 16 kDa.

1. Neutrophil activation

Hanks' balanced salt solution (HBSS) contained 0.4g KCl, 8g NaCl, 0.15g KH_2PO_4, 0.29g Na_2HPO_4-$7H_2O$, and 1 g glucose in 1 liter DDW.

1) Chemotactic activity for neutrophils

The sample for assay was placed into the lower compartment of a Boyden chamber, and a 3.0 μm filter (Millipore, trade name, available from Bedford, MA) was placed on it. Then human neutrophils suspended (2 x 10^6 cells/ml) in medium were placed on the filter in the upper compartment for the chemotaxis assay. The chambers were then incubated for 35 min at 37°C in a 5% CO_2 humidified atmosphere. The leading front distance of the cells from the surface of the filter was the mean value of the five microscopic fields. The chemotactic unit (CTU) was calculated by three parameters; fMet-Leu-Phe (FMLP, 10 nM)-stimulated maximal migration (FM), minimal migration without the chemotactic factor, termed random migration (RM), and migration induced by the sample (SM). CTU was defined as 100 x (SM-RM)/(FM-RM). In the protein purification of LECT2a, the total CTU in each purification step was calculated by multiplying the dilution of the sample for 50 CTU in the dilution-activity curve. The value of ED_{50} represents the concentration of chemotactic protein at half maximum-activity in the dose-response curve.

2) Release of lysosomal proteins (enzymes)

After preincubated at a concentration of 2 x 10^6 neutrophils/ml of HBSS for 10 min at 37°C, neutrophils were mixed with a solution, which contained LECT2a with 0.005% BSA in the presence or absence of cytochalasin B (5 μg/ml) and FMLP (100 nM).

a) Measurement of MPO activity

MPO activity was assayed as follows. The reaction mixture was consisted of neutrophil supernatant or cell homogenate, 1.7 mM tetrametnylbenzidine (TMB), 0.39 mM hydrogen peroxide, 84.2 mM sodium citrate buffer (pH 5.4), 7.2 % N,N'-dimethylformamide, phosphate buffered saline without calcium and magnecium (PBS(-)) and HBSS in a total volume of 200 μl per well of a 96 well-F-plate (#2-69620, available from Nunc, Denmark). Increase in absorbance at 650 nm in the reaction mixture at 37°C was measured with an automatic analyzer LFA-096 (trade name, available from Japan Spectroscopic Co., Tokyo, Japan) at 30 sec interval. One unit was defined as the activity producing an increase of 1.0 in absorbance at 650 nm/min/ml of original MPO preparation.

b) Measurement of β-glucuronidase (BGL) activity

BGL activity in supernatant and cell homogenate was assayed as follows. The reaction mixture consisted of neutrophil supernatant or cell homogenate, 1 mM 4-methylumbelliferyl-β-D-glucuronide, 0.05 % Triton X-100 and 0.1 M sodium acetate buffer (pH 3.5) in a total volume of a 40 μl in a 96 well-F-plate was incubated for 30 min at 37°C. A termination buffer (50 mM sodium glycine buffer (pH 10.4) containing 5 mM EDTA-disodium) was added. Fluorescence intensity was measured by an automatic fluorescence analyzer LFA-96F (trade name, available from Japan Spectroscopic Co., Tokyo, Japan) with wavelengths at 365 nm for excitation and 405 nm for emission. One unit of BGL activity was defined as the activity liberating 1 pmol of 4-methylumbelliferone/min/ml of the original BGL preparation.

c) Determination of lactoferrin (LF)

LF protein in supernatant and cell homogenate was measured as follows. A 100 μl of 0.05 % goat antiserum to human LF antibody diluted with reagent dilution buffer, 0.1 % BSA in PBS(-) (pH 7.4), was transferred to a 96 well-F-plate overnight at 4°C. After the plate was washed three times with PBS(-) containing 0.05 % Tween-20 washing buffer, 100 μl of diluted supernatant or cell homogenate was added to the plate. After kept for 30 min at room temperature, the plate was washed with the washing buffer three times, then a 100 μl of 0.1 % rabbit antiserum to human LF was added to the plate. After kept for 30 min at room temperature, the plate was washed three times with the washing buffer, and treated with a 100 μl of 0.025 % a peroxidase-labeled goat anti-rabbit Ig G. The plate was kept for 30 min at room temperature, washed three times with the washing buffer, and treated with a 200 μl of enzyme substrate in 0.05 M citrate buffer (pH 5.0) containing 0.025 % hydrogen peroxide and 0.04 % of o-phenylenediamide-di-hydrochloride for about 20 min at room temperature in the dark. Then, a 50 μl of 2.5 N sulfuric acid was added to the plate to

terminate the reaction, subsequently absorbance at 490 nm was measured with an automatic analyzer LFA-096, and LF content in supernatant or cell homogenate was determined.

3) Superoxide production of neutrophils

Superoxide production was measured as follows. Neutrophils suspension (2 x 10^6 cells/ml, 100 µl) and 0.066 mM of ferri cytochrome c were mixed in 96 well-F-Plate and kept for approximately 2 min. A sample, cytochalasin B (5 µg/ml) and FMLP (1000 nM) were subsequently added to the suspension and stood still for approximately 30 sec at 37°C. Superoxide production was determined by measuring the increase in absorbance at 546 nm at 0.269 min interval using the automatic analyzer LFA-096.

4) Adhesion of neutrophils

Neutrophils adhesion was measured as follows. Neutrophils suspension (2 x 10^6 cells/ml, 100 µl) was inoculated into a glass-rubber chamber. The sample containing LECT2a (10 µl), and FMLP (10^{-11} to 10^{-5}) were added to the chamber in a total volume of 150 µl. The chamber was incubated for 15 min at 37°C in a CO_2 incubator. After the incubation, non-adherent cells were aspirated and the cell concentration was counted. And then, the slide chamber was fixed with ethanol and stained with safulanin.

5) Membrane fluidity of neutrophils

After neutrophils and FITC-labeled succinyl concanavalin-A (FS-ConA) were incubated for 10 min at 37°C, PBS (-) was added to the mixture. The FS-ConA labeled neutrophils were prepared with HBSS at a concentration of 10^6 cells/ml, and then plated into a slide chamber. A solution of the leukocyte activating factor (10 µl) and FMLP (10 nM) was added to the cell suspension in a total volume 150 µl. The cell suspension was observed through an image analyzer (IMRAS) connected with Nikon inverted microscope. The membrane fluidity was analyzed with IMRAS processor.

Five activities of neutrophils carried out above procedures are summarized in Table 2.

Table 2

Summary of neutrophil activation with LECT2a						
Concentration of LECT2a (nM)	Release of Proteins (% Release*)			Superoxide Production (nmoles/ml)	Cell Adhesion (%)	Membrane Fluidity (sec/µm)
	MPO	BGL	LF			
0	24	45	14	12	88	36
0.01	25	43	14	14	88	38
0.1	27	46	17	13	86	33
1	26	46	16	12	89	36
10	24	44	17	11	90	39
100	25	44	16	15	93	42
1000	28	49	20	18	92	49
10000	35	52	25	25	95	53

* %Release=100 x S/ (S+H) .
S=extracellular enzyme activity (or protein content).
H=intracellular remained enzyme activity (or protein content).

2) Chemotactic activity of monocytes and lymphocytes

The procedures were almost same to those for neutrophils except for 5 µm Millipore filter instead of 3 µm, and except for 75-min incubation instead of 35-min incubation. The concentration of LECT2a given ED_{50} were 220 nM in monocytes and 430 nM in lymphocytes, respectively.

2. Amino acid sequencing of purified LECT2a

Ten micrograms of pyridyl ethylated LECT2a was digested with TPCK-trypsin (trade name, available from Worthington Biochemicals, Freehold, NJ) (E/S = 1:50, w/w) in 1.5 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM

$CaCl_2$ at 37°C for 8 hours. Peptides from the proteolytic digestion were separated by reverse-phase HPLC on a wide pore ODS column (0.46 x 25 cm) (trade name, available from J.T. Baker Research Products, Phillipsburg, NJ) with an acetonitrile gradient in 0.1% TFA. Automatic amino acid sequence analysis was carried out with a gas phase amino acid sequencer model 477A (trade name, available from Applied Biosystems, Foster City, CA).

3. Cloning of LECT2b and determination of nucleotide sequence of the cloned LECT2b

Polyadenylated [poly(A)+] RNA was prepared. For cloning, Poly(A)+ RNA (5 µg) from SKW-3 treated with PHA-P (50 µg/ml) was used as a template for synthesis of single-stranded cDNA using SuperScript™ reverse transcriptase (trade name, available from BRL, Grand Island, NY). Six 17-mer oligonucleotides for the amino acid sequences WAIICA (5'-oligonucleotide) were synthesized and four for HIENCD (3'-oligonucleotide) were synthesized. The 5'- and 3'-oligonucleotide sets were each used to prime the amplification of 45 ng of SKW-3 cDNA by PCR, which was carried out for 40 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min in a 50-µl reaction buffer. The amplified PCR products were separated on an agarose gel, blotted onto a Hybond-N+ nylon membrane (trade name, available from Amersham, Buckinghamshire, England), and subjected to hybridization with an internal nucleotide probe GATGTC/GCTA/GTGCTCT/CGATGGC/GTCT/CACT/AGC/GTATGCT/CTT corresponding to the amino acid sequence DVLCSDGSTVYAP. The PCR product detected by Southern hybridization was cloned into pUC19.

The PCR product was subjected to nucleotide sequence analysis by the dideoxynucleotide chain-termination method (available from United States Biochemicals, Cleveland, OH). A λgt10 library was constructed from liver mRNA and 1.3×10^6 independent clones were screened. Twelve positive clones were isolated and classified into two types by restriction endonuclease analysis. The sequence analysis of the longest clones of each type suggested that the two types of clone would be derived from an identical gene which had two poly(A)+ signals. The nucleotide sequence analysis revealed that the deduced amino acid sequences were 86% homologous to the LECT2a amino acid sequence.

4. Construction of the plasmid expressing a LECT2b-fusion protein

The cDNA fragments of LECT2b, which contained a EcoRI site at 5'-end and a HindIII site at 3'-end, were amplified by PCR with 5'-primer GGCGAATTCGAAAACCTGTATTTTCAGGGGCCCTGGGCTAATATATG and 3'-primer CGCAAGCTTTTACAGGTATGCAGTAG for 25 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The LECT2b cDNAs digested with EcoRI and HindIII restriction endnucleases were ligated into the EcoRI/-HindIII site of pMAL™-c expression vector (trade name, available from Biolab Inc.). This plasmid was named pMAL-TEV-LECT2b. An important features of this plasmid are that the recombinant fusion protein is inducible by IPTG treatment and has the recognition site of TEV protease (derived from Tabacco Etch Virus) between maltose-binding protein and LECT2b protein.

Induction of pGEX-Xa-LECT2b

The cDNA fragments of LECT2b, which contained a BamHI site at 5'-end and at 3'-end, were amplified by PCR with 5'-primer GCGGGATCCCCGGGCCATGGGCTAATAT and 3'-primer CGCGGATCCTTACAGGTATGCAGTAG for 25 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The LECT2b cDNAs digested with BamHI restriction endnucleases were ligated into the BamHI site of pGEX-3X expression vector (available from Pharmacia Inc.). This plasmid was named pGEX-Xa-LECT2b. An important features of this plasmid are that the recombinant fusion protein is inducible by IPTG treatment and has the recognition site of Xa protease between Glutation-S-transferase and LECT2b protein.

5. Transformation of E. coli by the vector expressing recombinant LECT2b

E. coli JM109 was transformed by pMAL-TEV-LECT2b, and resulted transformants were confirmed by DNA sequencing. The Mal-LECT2b strain (deposited at the NATIONAL INSTITUTE OF BIOSCIENCE AND HUMAN-TECHNOLOGY, Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, Japan under FERM P-14669 and transferred to international deposition under FERM BP-5302 on November 24, 1995 under the Budapest Treaty), which had the expression plasmid coding the precise LECT2b protein sequence, was selected.

Expression of LECT2b protein in animal cells

The pOLECT2b plasmid was digested with BglII and carried out by self-ligation. 5'-side of cDNA in the resulted plasmid deleted until up 5-bp from the predicted ATG by Exonuclease III. The deleted DNA was treated with klenow fragment and was ligated with PstI linkers. This deleted plasmid was digested with PstI and BglII, and PstI-BglII fragment of LECT2b was cloned into the PstI/BamHI site of pcDSRα296. This expression vector (pSRαLECT2b) was transfected

into CHO cells grown to about 50% confluence. Then, stable transformant cells (C1D8-1) (deposited at the NATIONAL INSTITUTE OF BIOSCIENCE AND HUMAN-TECHNOLOGY, Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, Japan under FERM P-14668 and transferred to international deposition under FERM BP-5301 on November 24, 1995 under the Budapest Treaty) highly producing LECT2b was obtained.

Determination of molecular size of LECT2b

The recombinant LECT2b protein expressed in CHO cells was metabolically labeled with ^{35}S-methionine and the proteins were detected by SDS-PAGE. The bands of LECT2b were detected in about 16 kDa in the position. These results indicate that molecular size of LECT2b was identical to that of LECT2a.

Determination of the activity

The activity of the leukocyte activating factor LECT2b was determined by the same procedures to those described for LECT2a. The some results were shown in Table 3.

Table 3

Summary of neutrophil activation with LECT2b						
Concentration of LECT2b (nM)	Release of MPO (% Release*)			Superoxide Production (nmoles/ml)		
	none	CB	CB+FMLP	none	CB	CB+FMLP
0	14.6	10.3	38.4	0	0	16.5
0.01	11.8	8.9	37.1	-1.7	0	17.7
0.1	11.8	8.9	37.5	0	0	16.3
1	13.2	10.2	33.9	1.2	0.6	18.3
10	12.6	10.0	33.9	0.7	0.8	22.9
100	12.7	10.9	36.9	0	0	21.9
1000	15.3	13.6	44.0	1.0	1.6	22.9
10000	21.8	17.2	55.3	0	1.0	20.3

*%Release=100 x S/ (S+H) .
S=extracellular MPO activity.
H=intracellular remained MPO activity.

LECT2a and LECT2b proteins are grouped in a novel cyto-kine/interleukin which activate immune cells. This shows that these proteins are useful for diagnosis, therapy and prediction of cancer by their immune reaction. Further, these proteins will be employed to cancer therapy. On the other hand, activation of neutrophils and other inflammation cells by these proteins will have relationship to chronic disease, suggesting that these proteins will be widely used for diagnosis, therapy and detection of disorder of cytokine network.

RAW SEQUENCE LISTING
PATENT APPLICATION

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: Kazuo SUZUKI et al.
(ii) TITLE OF INVENTION: NOVEL ACTIVATING FACTOR OF LEUKOCYTES
(iii) NUMBER OF SEQUENCES: 7
(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: 663-2, Shiigi
(B) STREET: Misaki-machi
(C) CITY: Isumi-gun
(D) STATE: Chiba-ken
(E) COUNTRY: JAPAN
(F) ZIP: 299-45
(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette, 3.50 inch, 1.44 MB storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: MS-DOS v.5
(D) SOFTWARE: Word Perfect 5.1
(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: JP 293233/1994
(B) FILING DATE: 28-NOV-1994
(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME:
(B) REGISTRATION NUMBER:
(C) PREFERENCE/DOCKET NUMBER:
(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE:
(B) TELEFAX:
(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 amino acids
(B) TYPE: amino acid
(C) STRANDNESS: single strand
(D) TOPOLOGY: lenear
(ii) MOLECULE TYPE: protein
(v) TYPE OF FRAGMENT: N-terminal fragment
(vi) ORIGINAL SOURCE:
(A) ORGANISM: human
(F) TISSUE TYPE: T-cell derived leukemia cells
(x) PUBLICATION INFORMATION:
(A) AUTHORS: Kazuo SUZUKI et al.
(B) TITLE OF INVENTION: NOVEL ACTIVATING FACTOR OF LEUKOCYTES
(K) RELEVANT RESIDUES IN SEQ ID NO:1: FROM 1 to 54

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Pro Trp Ala Ile Ile Cys Ala Gly Lys Ser Ser Asn Glu Ile Arg
1 5 10 15

Thr Cys Asp Gly His Gly Cys Gly Gln Tyr Thr Ala Gln Arg Asn Gln
20 25 30

Lys Leu His Gln Gly Val Asp Val Leu Cys Ser Asp Gly Ser Thr Val
35 40 45

Tyr Ala Pro Phe Xaa Gly
50

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDNESS: single strand
(D) TOPOLOGY: lenear
(ii) MOLECULE TYPE: protein
(v) TYPE OF FRAGMENT: intermediate fragment
(vi) ORIGINAL SOURCE:
(A) ORGANISM: human
(F) TISSUE TYPE: T-cell derived leukemia cells
(x) PUBLICATION INFORMATION:
(A) AUTHORS: Kazuo SUZUKI et al.
(B) TITLE OF INVENTION: NOVEL ACTIVATING FACTOR OF LEUKOCYTES
(K) RELEVANT RESIDUES IN SEQ ID NO:2: FROM 1 to 10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ile Met Gly Gln Glu Lys Pro Tyr Lys Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDNESS: single strand
(D) TOPOLOGY: lenear
(ii) MOLECULE TYPE: protein
(v) TYPE OF FRAGMENT: intermediate fragment
(vi) ORIGINAL SOURCE:
(A) ORGANISM: human
(F) TISSUE TYPE: T-cell derived leukemia cells
(x) PUBLICATION INFORMATION:
(A) AUTHORS: Kazuo SUZUKI et al.
(B) TITLE OF INVENTION: NOVEL ACTIVATING FACTOR OF LEUKOCYTES
(K) RELEVANT RESIDUES IN SEQ ID NO:3: FROM 1 to 9

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ile Ser Gly Gly Gly Phe Cys Ile Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDNESS: single strand
(D) TOPOLOGY: lenear
(ii) MOLECULE TYPE: protein
(v) TYPE OF FRAGMENT: intermediate fragment
(vi) ORIGINAL SOURCE:
(A) ORGANISM: human
(F) TISSUE TYPE: T-cell derived leukemia cells
(x) PUBLICATION INFORMATION:
(A) AUTHORS: Kazuo SUZUKI et al.
(B) TITLE OF INVENTION: NOVEL ACTIVATING FACTOR OF LEUKOCYTES
(K) RELEVANT RESIDUES IN SEQ ID NO:4: FROM 1 to 5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Tyr Lys Gly Ser Ile
1 5

(2) INFORMATION FOR SEQ ID NO: 5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDNESS: single strand
(D) TOPOLOGY: lenear
(ii) MOLECULE TYPE: protein
(v) TYPE OF FRAGMENT: intermediate fragment
(vi) ORIGINAL SOURCE:
(A) ORGANISM: human
(F) TISSUE TYPE: T-cell derived leukemia cells
(x) PUBLICATION INFORMATION:
(A) AUTHORS: Kazuo SUZUKI et al.
(B) TITLE OF INVENTION: NOVEL ACTIVATING FACTOR OF LEUKOCYTES
(K) RELEVANT RESIDUES IN SEQ ID NO:5: FROM 1 to 20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Val Tyr Pro Gly Ile Gln Ser His Ile His Ile Glu Asn Xaa Asp Leu
1 5 10 15

Ser Asp Pro Thr
20

(2) INFORMATION FOR SEQ ID NO: 6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 151 amino acids
(B) TYPE: amino acid

(C) STRANDNESS: single strand
(D) TOPOLOGY: lenear
(ii) MOLECULE TYPE: protein
(v) TYPE OF FRAGMENT: intermediate fragment
(vi) ORIGINAL SOURCE:
(A) ORGANISM: human
(F) TISSUE TYPE: T-cell derived leukemia cells
(x) PUBLICATION INFORMATION:
(A) AUTHORS: Kazuo SUZUKI et al.
(B) TITLE OF INVENTION: NOVEL ACTIVATING FACTOR OF LEUKOCYTES
(K) RELEVANT RESIDUES IN SEQ ID NO:6: FROM 1 to 151

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Phe Ser Thr Lys Ala Leu Leu Leu Ala Gly Leu Ile Ser Thr Ala
 1               5                  10                  15
Leu Ala Gly Pro Trp Ala Asn Ile Cys Ala Gly Lys Ser Ser Asn Glu
            20                  25                  30
Ile Arg Thr Cys Asp Arg His Gly Cys Gly Gln Tyr Ser Ala Gln Arg
        35                  40                  45
Ser Gln Arg Pro His Gln Gly Val Asp Val Leu Cys Ser Ala Gly Ser
    50                  55                  60
Thr Val Tyr Ala Pro Phe Thr Gly Met Ile Val Gly Gln Glu Lys Pro
65                  70                  75                  80
Tyr Gln Asn Lys Asn Ala Ile Asn Asn Gly Val Arg Ile Ser Gly Arg
                85                  90                  95
Gly Phe Cys Val Lys Met Phe Tyr Ile Lys Pro Ile Lys Tyr Lys Gly
            100                 105                 110
Pro Ile Lys Lys Gly Glu Lys Leu Gly Thr Leu leu Pro Leu Gln Lys
        115                 120                 125
Val Tyr Pro Gly Ile Gln Ser His Val His Ile Glu Asn Cys Asp Ser
    130                 135                 140
Ser Asp Pro Thr Ala Tyr Leu
145                 150
```

(2) INFORMATION FOR SEQ ID NO: 7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1092 nucleic acids
(B) TYPE: nucletotides
(C) STRANDNESS: double strand
(D) TOPOLOGY: lenear
(ii) MOLECULE TYPE: cDNA
(vi) ORIGINAL SOURCE:
(A) ORGANISM: human
(F) TISSUE TYPE: liver
(x) PUBLICATION INFORMATION:
(A) AUTHORS: Kazuo SUZUKI et al.
(B) TITLE OF INVENTION: NOVEL ACTIVATING FACTOR OF LEUKOCYTES
(K) RELEVANT RESIDUES IN SEQ ID NO:7: FROM 1 to 1092

Specificity of the sequence
Mark describing the specificity: 5'UTR
Localization: 1 ... 200
Method for determination of the specificity: P
Mark describing the specificity: 3'UTR
Localization: 657 ... 1092
Method for determination of the specificity: P
Mark describing the specificity: CDS
Localization: 201 ... 656
Method for determination of the specificity: P
Mark describing the specificity: mutaion
Localization: replace (372, " a")
replace (748, " g")
replace (961, " c")
replace (967, " c")
Method for determination of the specificity: E
Mark describing the specificity: polyA signal
Localization: 684 ... 689
1060 ... 1065
Method for determination of the specificity: E

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
AAATCAAATA GCTATCCATG GAATATTAGA ACTTGACTTG CTCCATCCTC      50
TTAAACTTTT TGTGTCTCAC ACTAAAGAAA TGAGAGATGC AGAATTCTAA     100
GGCTAAATAG CTAGGAAGTA TTCATTCAAA CTTGAATATC TTCAAAGAGA     150
GTGTGGGGGC AACTCTAATC AGAGGAAGAA ACTAAAGGAA GTAAAACCAG     200
ATG TTT TCC ACC AAA GCC CTC CTT TTG                        227
Met Phe Ser Thr Lys Ala Leu Leu Leu
 1               5
GCT GGT CTG ATT TCT ACC GCA CTG GCA GGG CCA TGG GCT AAT    269
Ala Gly Leu Ile Ser Thr Ala Leu Ala Gly Pro Trp Ala Asn
 10                  15                  20
ATA TGT GCT GGC AAG TCT TCC AAT GAG ATC CGG ACG TGT GAC    311
Ile Cys Ala Gly Lys Ser Ser Asn Glu Ile Arg Thr Cys Asp
      25                  30                  35
CGC CAT GGC TGT GGA CAG TAC TCT GCT CAA AGA AGT CAG AGG    353
Arg His Gly Cys Gly Gln Tyr Ser Ala Gln Arg Ser Gln Arg
          40                  45                  50
CCT CAC CAG GGT GTG GAC GTC TTG TGC TCT GCT GGA TCT ACT    395
Pro His Gln Gly Val Asp Val Leu Cys Ser Ala Gly Ser Thr
              55                  60                  65
GTG TAC GCA CCA TTC ACT GGA ATG ATT GTG GGC CAG GAG AAA    437
Val Tyr Ala Pro Phe Thr Gly Met Ile Val Gly Gln Glu Lys
                  70                  75
CCT TAT CAA AAC AAG AAT GCT ATC AAT AAT GGT GTT CGA ATA    479
Pro Tyr Gln Asn Lys Asn Ala Ile Asn Asn Gly Val Arg Ile
 80                  85                  90
TCT GGA AGA GGT TTT TGT GTC AAA ATG TTC TAC ATT AAG CCA    521
Ser Gly Arg Gly Phe Cys Val Lys Met Phe Tyr Ile Lys Pro
      95                  100                 105
ATT AAG TAT AAA GGT CCT ATT AAG AAG GGA GAA AAA CTT GGA    563
Ile Lys Tyr Lys Gly Pro Ile lys Lys Gly Glu Lys Leu Gly
          110                 115                 120
```

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ACT CTA TTG CCC TTG CAG AAA GTT TAT CCT GGC ATA CAA TCG    605
Thr Leu leu Pro Leu Gln Lys Val Tyr Pro Gly Ile Gln Ser
            125                 130                 135
CAT GTG CAC ATT GAA AAC TGT GAC TCG AGT GAC CCT ACT GCA    647
His Val His Ile Glu Asn Cys Asp Ser Ser Asp Pro Thr Ala
                140                 145
TAC CTG                                                   653
Tyr Leu
150
TAAATCGAAG GCCAATGGTC AGATCTTCAA AATAAAAAGT CATCTTAAAA     703
ACCTGGATGC ATACCCTTCT CTTCAAGAAA TTTGTGTTCA CAAAAGAAAA     753
ATGCATGAAG GGATGGATAC CCCATTTTCC ATGACATGAT TATTACACAT     803
TGCATGCCTG TATCAAAACA TCTCACGTAC CTCATAAACA TATACACCTA     853
TGTACCCACA AAAATTTTTT AATTAAAAAA AGGAAATTTG AGTTTAAATA     903
GAAACATGAT AAATGCAAGA AAGAAAACAT TTTGATTTTA ACTCATTGTC     953
ACTCTGATGT TCATGTGAAC TGGTTGCTTC GGGCTCTTTG ATCTGTCACC    1003
TATGGAATCT GAGTGGTTTT ATTTTTTAGA TTTCTCAGTC CCAAAGATCT    1053
AAGATAAATA AACAAGAGAA CTTAAAAAAA AAAAAAAAA                1092
```

Claims

1. Human LECT2a polypeptide having amino acid sequence in the order shown in Sequence ID NOs. from 1 to 5 in the sequence table, which did not include other proteins/peptides.

2. Human LECT2b polypeptide having amino acid sequence in the order shown in Sequence ID NO: 6 in the sequence table, which did not include other proteins/peptides.

3. DNA encoding LECT2b amino acid sequence in the order shown in Sequence ID NO: 6 in the sequence table.

4. DNA according to Claim 3, wherein said DNA has nucleotide sequence shown in Sequence ID NO: 7 in the sequence table.

5. Recombinant plasmids containing DNA encoding human LETCT2b having an amino acid sequence shown in Sequence ID NO: 6 in the sequence table.

6. The recombinant plasmids according to Claim 5, wherein said plasmid is a plasmid pMAL-TEV-LECT2b constructed by ligation of the nucleotide sequence encoding LECT2b protein at down-stream of maltose-binding protein region in pMal-c vector to induce fused protein by IPTG treatment and having a TEV protease recognition site which located between maltose binding protein and LECT2b.

7. The recombinant plasmids according to Claim 5, wherein said plasmid is a plasmid pGEX-Xa-LECT2b constructed by ligation of the nucleotide sequence encoding LECT2b protein at down-stream of glutathione-S-transferase region in pGEX vector to induce fused protein by IPTG treatment and having a Xa protease recognition site which located between glutathione-S-transferase and LECT2b.

8. The recombinant plasmids according to Claim 5, wherein the nucleotide sequence encoding human LECT2b is regulated with SRα promotor.

9. Transformed cells transfected with plasmid constructs which contain human LECT2b gene encoding amino acid sequence shown in the sequence ID. NO.: 6 in the sequence table.

10. The transformed cells according to Claim 9, wherein the transformed cells are obtained by transforming into the cells selected from the group consisting of E. coli, yeast, insect cells, Chinese hamster CHO cells, monkey CVI

cells, monkey CVI/293 cells, mouse fibroblast, mouse C127 cells, mouse 3T3 cells, mouse L-929 cells, human HeLa cells and human SKW-3 cells.

FIG. 1